Pulmonary Pharmacology & Therapeutics 45 (2017) 210–217

Contents lists available at ScienceDirect

Pulmonary Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/ypupt

The effect of tiotropium in combination with olodaterol on house dust mite-induced allergic airway disease☆

Gerrit John-Schuster [a, *, 1], Stan de Kleijn [a, 1], Yolanda van Wijck [a], Veerle Kremer [a], Hermelijn H. Smits [b], Michael P. Pieper [c], Pieter S. Hiemstra [a], Christian Taube [a]

[a] Department of Pulmonology, Leiden University Medical Center, Albinusdreef 2, 2333, ZA Leiden, The Netherlands
[b] Department of Parasitology, Leiden University Medical Center, Albinusdreef 2, 2333, ZA Leiden, The Netherlands
[c] Boehringer Ingelheim, Birkendorfer Str. 65, 88400, Biberach, Germany

ARTICLE INFO

Article history:
Received 30 January 2017
Received in revised form
12 June 2017
Accepted 29 June 2017
Available online 5 July 2017

Keywords:
Asthma
House dust mite
Airway inflammation
Remodeling
Tiotropium

Chemical compounds:
Tiotropium bromide (PubChem CID: 5487426)
Olodaterol (PubChem CID: 11711522)

ABSTRACT

One of the major goals of asthma therapy is to maintain asthma control and prevent acute exacerbations. Long-acting bronchodilators are regularly used for the treatment of asthma patients and in clinical studies the anti-cholinergic tiotropium has recently been shown to reduce exacerbations in patients with asthma. So far it is unclear how tiotropium exerts this effect. For this purpose, we designed an allergen-driven rechallenge model of allergic airway inflammation in mice, to assess the effectiveness of tiotropium and the long-acting β-2 adrenoceptor agonist olodaterol on allergen-induced exacerbations of airway disease.

Female C57BL/6J mice were sensitized intranasally (i.n.) with 1 μg of house dust mite (HDM) extract followed by a challenge regime (5 consecutive days 10 μg HDM extract i.n.) after one week. Mice were exposed to a secondary challenge five weeks after sensitization and were treated i.n. with different concentrations of tiotropium or olodaterol (1, 10 and 100 μg/kg) or a combination thereof (10 μg/kg each) prior to and during the secondary challenge period. Three days after the last challenge, bronchoalveolar lavage (BAL) fluid and lung tissue were collected for flow cytometry and histologic analysis, respectively.

Secondary challenge with HDM extract strongly induced allergic airway disease reflected by inflammatory cell infiltration and goblet cell metaplasia. Treatment with tiotropium, but not with olodaterol reduced tissue inflammation and goblet cell metaplasia in a dose-dependent manner. The combination of tiotropium and olodaterol was more effective in significantly reducing tissue inflammation compared to tiotropium treatment alone, and also led to a decrease in BAL cell counts.

These data suggest that in a model of relapsing allergic airway disease tiotropium directly prevents exacerbations by reducing inflammation and mucus production in the airways. In addition, the combination of tiotropium and olodaterol exerts synergistic effects.

1. Introduction

Asthma is a chronic respiratory disease most commonly caused by hypersensitivity to a variety of allergens and is one of the leading causes of chronic disease worldwide [1]. The disease pathogenesis is characterized by complex airway inflammatory and remodeling processes that lead to airway hyperresponsiveness and various degrees of reversible airflow limitation. Current therapies preferentially consist of a combination of inhaled corticosteroids and inhaled long-acting β-2 adrenoceptor agonists (LABA) and are

Abbreviations: HDM, house dust mite; OVA, ovalbumin; BAL, bronchoalveolar lavage; LABA, long-acting β-2 adrenoceptor agonist.

☆ Part of this work was presented in abstract form at the International Conference of the American Thoracic Society 2015 and published as a conference abstract: de Kleijn S, van Wijck Y, Kremer V, Smits H, Pieper M, Hiemstra P, Taube C. Tiotropium inhibits airway inflammation and goblet cell metaplasia in a house dust mite (HDM)-induced rechallenge model of allergic airway disease. Am J Respir Crit Care Med 191 (2015) A4272.

* Corresponding author. Department of Pulmonology, Leiden University Medical Center, Albinusdreef 2, 2333, ZA Leiden, The Netherlands.
E-mail address: G.John-Schuster@lumc.nl (G. John-Schuster).

[1] Equal contribution.

http://dx.doi.org/10.1016/j.pupt.2017.06.010

beneficial in controlling symptoms and airway inflammation, but have little effect on airway remodeling. However, the majority of asthma morbidity occurs in patients suffering from a severe form of the disease (up to 10% of all asthmatics) with recurring symptoms and exacerbations that are poorly controlled despite the use of standard combination therapy [2].

Recent clinical studies have focused on the option of adding a second long-acting bronchodilator, the anticholinergic agent tiotropium that has been used in the (maintenance and exacerbation) treatment of chronic obstructive pulmonary disease (COPD) for the past years [3–5]. Tiotropium is a muscarinic receptor antagonist that is kinetically selective for M3 receptors [6]. Muscarinic receptor signaling in the airways is primarily induced via the parasympathetic neurotransmitter acetylcholine released by both neuronal and non-neuronal cells including lung structural and inflammatory cells [7]. Signaling induces bronchoconstriction and mucus production by acting on smooth muscles and mucus-secreting cells in the central airways [8–11]. Blocking the receptor using therapeutic muscarinic receptor antagonists results in smooth muscle relaxation and reduced mucus production [12].

Besides its function as a bronchodilator, tiotropium has been demonstrated to reduce signs of allergen-induced airway inflammation in animal models [13–15], and interestingly also in combination with a novel LABA, olodaterol [16]. However, most of these studies were performed with allergens irrelevant to the human situation and so far not in secondary challenge models, in which airway inflammation has been established. Therefore, the aim of the current study was to determine the effect of treatment with tiotropium or olodaterol on allergen-induced exacerbation of airway inflammation and remodeling. To achieve this, a rechallenge model of allergic airway inflammation in mice mimicking allergen-induced exacerbations of the disease was utilized. In addition, a potential synergistic effect of a combination treatment of tiotropium and olodaterol was explored in this model.

2. Material and methods

2.1. Animals and maintenance

8-10 weeks old pathogen-free female C57BL/6J mice (Charles River, 's-Hertogenbosch, The Netherlands) were housed in rooms maintained at constant temperature and humidity with a 12-h light cycle. Animals were allowed food and water *ad libitum*.

All animal procedures were approved by the local animal ethics committee of the Leiden University Medical Center (license number 13185, Dierexperimentencommissie Academisch Ziekenhuis Leiden) and were conducted under strict governmental and international guidelines in accordance with EU Directive 2010/63/EU.

2.2. Treatment protocol

Mice were sensitized by intranasal administration of 1 μg house dust mite extract (HDM; Greer, Lenoir, NC, USA) in 50 μl PBS on day 0 (week 1) and challenged intranasally with 10 μg HDM extract in 50 μl PBS once daily on days 7–11 (week 2). After a recovery period of three weeks, animals were rechallenged intranasally with 10 μg HDM extract once daily on days 35–39 (week 6). Prior to and during the second allergen challenge period, animals were treated daily by intranasal administration of 1, 10 and 100 μg/kg bodyweight (BW) tiotropium (Boehringer Ingelheim; dissolved in PBS), olodaterol (Boehringer Ingelheim; dissolved in PBS) or a combination thereof (both compounds at 10 μg/kg each) starting on day 34, 24 h before the first rechallenge and on days 35–39, 1 h before the challenge. Intranasal administration was performed under isoflurane anesthesia (3%, 0.6 L/min). Control animals received 50 μl PBS intranasally during sensitization, challenge and rechallenge. For a detailed protocol outline, see Fig. 1.

Three days after the last challenge, mice were euthanized with sodium pentobarbital and tracheostomized. All animals were lavaged, the left lung was removed for flow cytometry analysis, and the right lung was fixed in paraformaldehyde (PFA; see below).

2.3. Bronchoalveolar lavage (BAL) and lung single cell suspension

BAL fluid and lung single cell suspensions were obtained to determine lung inflammatory cell recruitment. BAL was performed by instilling the lungs with 3 × 1 ml aliquots of sterile PBS (Braun). Afterwards, cells were spun down at 400 g and resuspended in 0.5% (w/v) BSA (Sigma)/2 mM EDTA (Invitrogen) FACS buffer. Total cell counts were determined in a hemocytometer. Remaining cells were subjected to flow cytometry analysis (see below).

For single-cell suspensions of whole lung tissue, lungs were perfused with sterile PBS via the right ventricle to clear leukocytes and erythrocytes from the pulmonary circulation. Lung homogenization was performed via enzymatic digestion and mechanical dissociation steps using collagenase (1 mg/ml; Calbiochem)/DNase (20 U/ml; Sigma) and 70 μm cell strainers (Corning) followed by red blood cell lysis. Single-cell suspensions were subjected to flow cytometry analysis (see below).

2.4. Flow cytometry

For flow cytometry, cells were stained with live/dead stain Aqua (Invitrogen) and fixed in 1.9% w/v formaldehyde (Merck). The following antibodies were used to distinguish different inflammatory cell populations (neutrophils, macrophages, eosinophils, DCs, B and T cells) in the BAL fluid: Ly-6G/Ly-6C (Gr-1)-FITC (RB6-8C5,

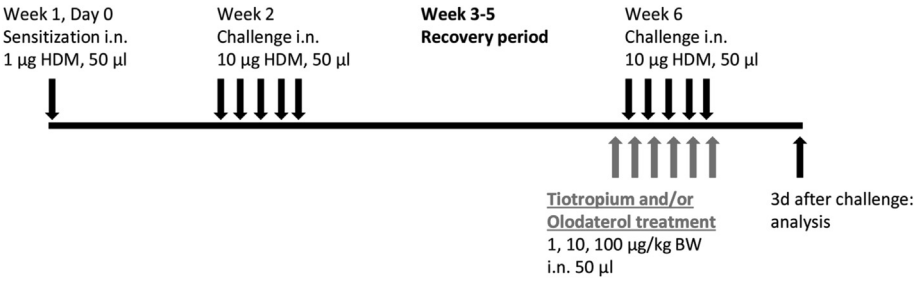

Fig. 1. Rechallenge model of allergic airway inflammation. Mice were sensitized by intranasal administration of 1 μg house dust mite extract (HDM; Greer, Lenoir, NC, USA) in 50 μl PBS on day 0 (week 1) and challenged intranasally with 10 μg HDM extract in 50 μl PBS once daily on days 7–11 (week 2). After a recovery period of three weeks, animals were rechallenged intranasally with 10 μg HDM extract once daily on days 35–39 (week 6). Prior to and during the second allergen period, animals were treated daily by intranasal administration of 1, 10 and 100 μg/kg bodyweight (BW) tiotropium (Boehringer Ingelheim; dissolved in PBS), olodaterol (Boehringer Ingelheim; dissolved in PBS) or a combination thereof (both compounds at 10 μg/kg each) starting on day 34, 24 h before the first rechallenge and on days 35–39, 1 h before the challenge.

BD Biosciences), Siglec-F-PE (E50-2440, BD Biosciences), CD3-PerCP-eFluor710 (17A2, eBioscience), CD11b-PE-Cy7 (M1/70, eBioscience), MHCII (I-A/I-E)-APC (M5/114.15.2, eBioscience), CD45R (B220)-APC-eFluor780 (RA3-6B2, eBioscience), and CD11c-V450 (HL3, BD Biosciences). Cells were measured on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) and analysis was performed using FlowJo (v7.6.5) software (Tree Star, Ashland, OR). Briefly, single, alive cells were separated into lymphocytes and non-lymphocytes based on FSC/SSC characteristics of the different populations. In the non-lymphocyte gate, eosinophilic granulocytes were identified as $CD11c^{-}Siglec\text{-}F^{+}$.

2.5. Histology

Lung tissue was fixed by intratracheal instillation of PBS buffered 3.9% w/v paraformaldehyde (PFA) and embedded into paraffin for hematoxylin-eosin (H&E) or periodic acid-Schiff (PAS) staining according to standard protocols. For determination of lung histopathology, tissue sections were examined in blinded fashion on a BX40 Olympus microscope. Peribronchial inflammation was scored on a scale from 0 to 4. PAS-positive goblet cells were quantified per 1 mm of basement membrane using ImageJ software.

2.6. Statistical analysis

Results are given as mean values ± SEM. One-way ANOVA following Dunnett post test was used for all studies. Analyses were conducted using GraphPad Prism 6 software (GraphPad Software, La Jolla, USA), and differences with $p < 0.05$ were considered statistically significant. Synergy between the effects of tiotropium and olodaterol was calculated by comparing the calculated sum of the effects of the individual drugs to the measured effect of the combination treatment using a Student's *t*-test, with $p < 0.05$ indicating a statistically significant difference showing synergistic activity.

3. Results

3.1. Tiotropium reduces allergic airway inflammation and remodeling after HDM rechallenge in a dose dependent manner

Tiotropium has been demonstrated to reduce exacerbations in patients with asthma [17]. To determine the effect of tiotropium monotherapy on relapsing allergic airway inflammation, mice were challenged with HDM extract in 2 periods of 5 days with three weeks recovery in between challenges (see Fig. 1). Prior to and during the second challenge, mice were treated with 1, 10 and 100 μg/kg BW of tiotropium. Treatment had no effect on the number of total cells and eosinophils in BAL fluid (Fig. 2A). However, when analyzing lung tissue, treatment with tiotropium resulted in a dose dependent decrease of airway inflammation and PAS-positive cells, and this reduction was statistically significant compared to the non-treated animals at the highest dose of tiotropium (Fig. 2B–E).

3.2. Olodaterol monotherapy does not reduce allergic airway inflammation after HDM rechallenge

Besides its bronchodilatory function, olodaterol has also been described to have anti-inflammatory properties [18]. To determine whether olodaterol is able to inhibit allergen-induced exacerbations, mice were treated prior to and during the second challenge with different doses of olodaterol. Treatment with olodaterol had no effect on airway inflammation in both BAL and lung tissue (Fig. 3A–C). In addition, treatment with olodaterol did not affect the number of PAS-positive cells in this model (Fig. 3D–E).

3.3. A combination of tiotropium and olodaterol has a synergistic effect on airway inflammation in the HDM rechallenge model

To assess if tiotropium and olodaterol have synergistic effects when used in combination, mice were treated with an intermediate dose of tiotropium (10 μg/kg BW) in combination with an intermediate dose of olodaterol (10 μg/kg BW). Both compounds did not reduce airway inflammation with these doses when applied in monotherapy (Figs. 2 and 3). In contrast, treatment with a combination of olodaterol and tiotropium significantly and synergistically reduced total cell and eosinophil counts in BAL fluid (Fig. 4A). In addition, a significant reduction in tissue inflammation following combination treatment was detectable and was found to be resulting from an additive effect rather than a synergistic effect of the combination (Fig. 4B–C). However, goblet cell metaplasia was not significantly reduced in tiotropium/olodaterol-treated mice (Fig. 4D–E).

4. Discussion

Our study aimed at investigating the effects of a long-acting anti-cholinergic and a long-acting beta-2-agonist on allergen induced asthma exacerbations in an allergen-induced rechallenge model of allergic airway inflammation. In the present study we show that treatment with the anticholinergic tiotropium inhibits tissue inflammation and goblet cell metaplasia induced by rechallenge with allergen, in contrast to treatment with the long-acting beta-2-agonists olodaterol. Interestingly, when used in combination, tiotropium and olodaterol showed a synergistic effect on the reduction of airway inflammation, specifically BAL total cell and eosinophil numbers.

Tiotropium has been the focus of many clinical studies due to its successful use in maintenance and exacerbation treatment of COPD [3–5]. In comparative asthma studies with adrenergic agents, tiotropium was found to be as effective as (or non-inferior to) salmeterol in improving symptoms and lung function when added to an inhaled glucocorticoid in patients with inadequately controlled asthma [19–21]. Furthermore, the addition of once-daily tiotropium to standard asthma combination treatment not only improved lung function [17,22], but also significantly increased the time to the first severe exacerbation, with an overall risk reduction of 21% [17]. Based on these results, tiotropium was recently approved as add-on treatment in long-term maintenance therapy of asthma [1,23,24]. However, it is currently unclear how tiotropium exerts its effects on asthma exacerbations.

In the present study we provide additional evidence that tiotropium has anti-inflammatory effects in allergen-induced exacerbations of airway disease. Tiotropium has been shown to reduce allergic airway inflammation in different models of allergic airway disease. Indeed, in guinea pigs tiotropium was similarly effective as the corticosteroid budesonide in inhibiting or reducing several aspects of airway inflammation and remodeling following challenges with ovalbumin (OVA) [25,26]. Similar results were found in different murine models of OVA-induced airway inflammation. Again, treatment with tiotropium significantly reduced airway inflammation and remodeling [13,14]. Buels et al. further identified that non-bronchodilating anti-inflammatory mechanisms of tiotropium were responsible for reducing airway hyperreactivity in a guinea pig OVA model of allergic asthma via inhibition of eosinophil accumulation in the lungs [27]. Treatment effects on inflammation and mucus hypersecretion in mice were also comparable to the corticosteroid dexamethasone, both during initiation and relapse of the disease induced by allergen rechallenge 90 days after the last challenge [15].

However, tiotropium effects have mainly been studied using

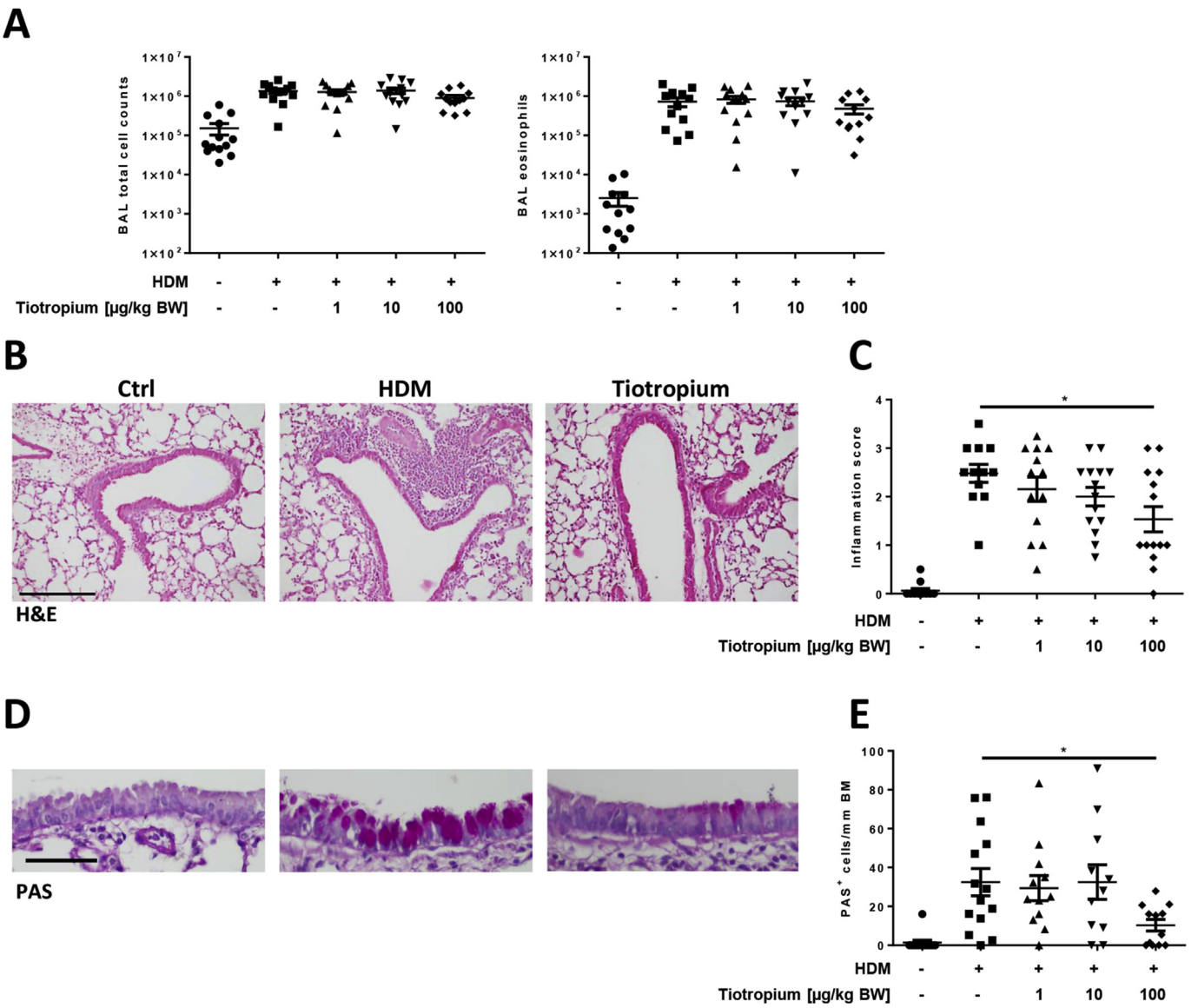

Fig. 2. A high dose of tiotropium treatment inhibits airway inflammation and goblet cell metaplasia. Mice were treated with 3 different doses of tiotropium (1, 10 and 100 µg/kg BW) prior to and during the second allergen challenge period. **A** Total inflammatory cells in BAL fluid were determined in a hemocytometer; absolute number of eosinophils were measured using FACS analysis by gating on $CD11c^{-}Siglec\text{-}F^{+}$ cells; **B** representative micrographs of H&E-stained lung tissue sections from naïve, HDM allergic and HDM allergic mice treated with tiotropium, scale bar 200 µm; **C** quantification of lung tissue inflammation; **D** representative micrographs of PAS-stained lung tissue sections from naïve, HDM allergic and HDM allergic mice treated with tiotropium, scale bar 50 µm; **E** number of PAS-positive cells per mm of basal membrane. Graphs show mean ± SEM; n = 12 mice; $^{*}p < 0.05$.

ovalbumin as allergen or in more prophylactic treatment approaches, such as starting treatment before the first allergen exposure. In the present study we utilized the human relevant allergen HDM [28]. In addition we made use of a secondary exposure in which mice had already developed allergic airway inflammation until they are challenged again, mimicking allergen-induced exacerbation of airway disease. We observed that tiotropium inhibited airway inflammation and goblet cell metaplasia after HDM rechallenge, whereas BAL cell counts were not affected, potentially due to ongoing and increased clearance of inflammatory cells via the airway lumen.

The anti-inflammatory effects of tiotropium are thought to be directly related to inhibition of acetylcholine-mediated production of pro-inflammatory factors and the subsequent accumulation of inflammatory cells in the lung in response to allergens. Both inflammatory and epithelial cells have been described to induce pro-inflammatory responses via acetylcholine and muscarinic receptors expressed throughout the lung [29–31], and tiotropium was able to inhibit these responses. While these results point towards an inhibition of remodeling as a consequence of reduced inflammation, it might also be caused by direct inhibitory effects on bronchoconstriction. This is supported by a recent study demonstrating that repeated methacholine challenge in patients with asthma induced airway remodeling, without an effect on inflammation [32]. Similarly, knockout of the M3 receptor in mice reduced allergen-induced parameters of airway remodeling, such as goblet cell metaplasia, smooth muscle thickening and collagen deposition [33], but did not affect inflammation including eosinophil numbers and Th2 cytokine levels. Furthermore, tiotropium has been shown to directly inhibit and reverse IL-13-induced goblet cell metaplasia and mucus (MUC5AC) production in primary human airway epithelial cells (indicating a direct effect of non-neuronal acetylcholine in this process) [34]. This might also help to explain the significant effects on remodeling observed in our study.

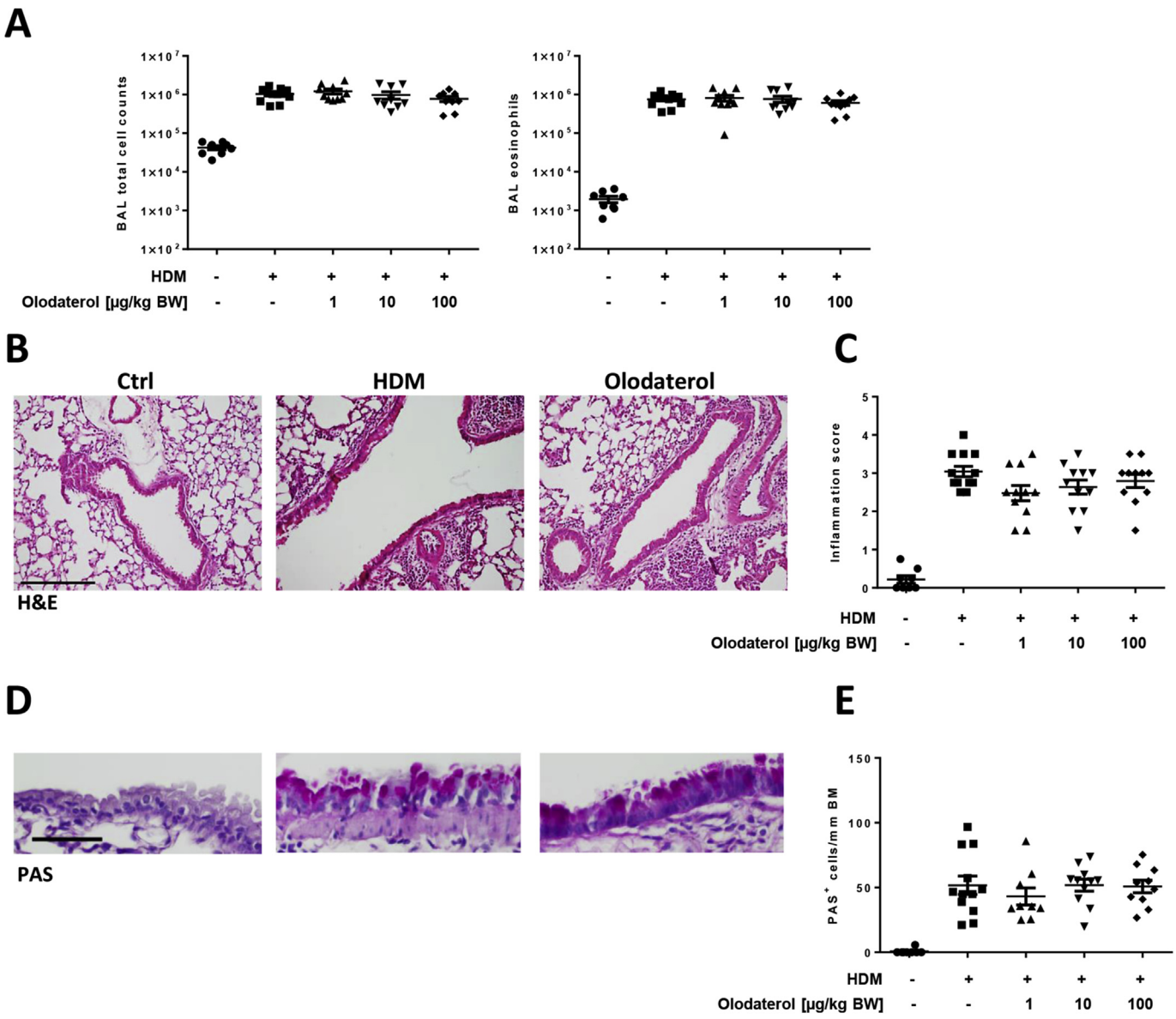

Fig. 3. Olodaterol does not inhibit airway inflammation and goblet cell metaplasia. Mice were treated with 3 different doses of olodaterol (1, 10 and 100 µg/kg BW) prior to and during the second allergen challenge period. **A** Total inflammatory cells in the BAL fluid were determined in a hemocytometer; absolute number of eosinophils were measured using FACS analysis; **B** representative micrographs of H&E-stained lung tissue sections from naïve, HDM allergic and HDM allergic mice treated with olodaterol, scale bar 200 µm; **C** quantification of lung tissue inflammation; **D** representative micrographs of PAS-stained lung tissue sections from naïve, HDM allergic and HDM allergic mice treated with olodaterol, scale bar 50 µm; **E** number of PAS-positive cells per mm of basal membrane. Graphs show mean ± SEM; n = 12 mice; $^{*}p < 0.05$.

Interestingly, in our HDM rechallenge model we observed a more significant treatment effect on airway inflammation combined with a reduction in BAL cell counts when a lower dose of tiotropium (10 µg/kg BW) was combined with olodaterol (10 µg/kg BW), a novel and highly selective LABA approved for long-term, once-daily maintenance bronchodilator treatment in COPD patients [35]. Olodaterol exerts its pharmacological effects by binding and activating beta-2 adrenoceptors in the airways, which stimulates intracellular adenyl cyclase, an enzyme that mediates the synthesis of cyclic adenosine monophosphate (cAMP). Elevated levels of cAMP induce bronchodilation by relaxation of airway smooth muscle cells [35]. Beneficial therapeutic effects of tiotropium/olodaterol combination treatment have especially been described for COPD [36,37]. In (moderate to very severe) COPD patients, the combination treatment improved lung function and health-related quality of life compared to monotherapy with either tiotropium or olodaterol alone [38–40]. The benefits are related to the maximized bronchodilating effect mediated by β-2 adrenoceptor-dependent intracellular cAMP increase combined with the competitive antagonism of M3 receptors [36]. Thus, a combination therapy of tiotropium/olodaterol (Stiolto Respimat) has recently been approved for the maintenance of COPD [41,42].

In a guinea pig model of allergic asthma (OVA), tiotropium synergistically enhanced the bronchoprotective effect of olodaterol [16]. However, Smit et al. did not observe any treatment effect on inflammatory cell infiltration in the airways, which is in contrast to our results obtained after combination treatment. Interestingly, in pulmonary fibroblasts from asthmatic and non-asthmatic subjects a combination of tiotropium and olodaterol restored intracellular cAMP levels beyond levels induced by olodaterol alone and thereby significantly reduced IL-1β-induced IL-8 and IL-6 release compared to tiotropium or olodaterol treatment alone [18]. However, Costa

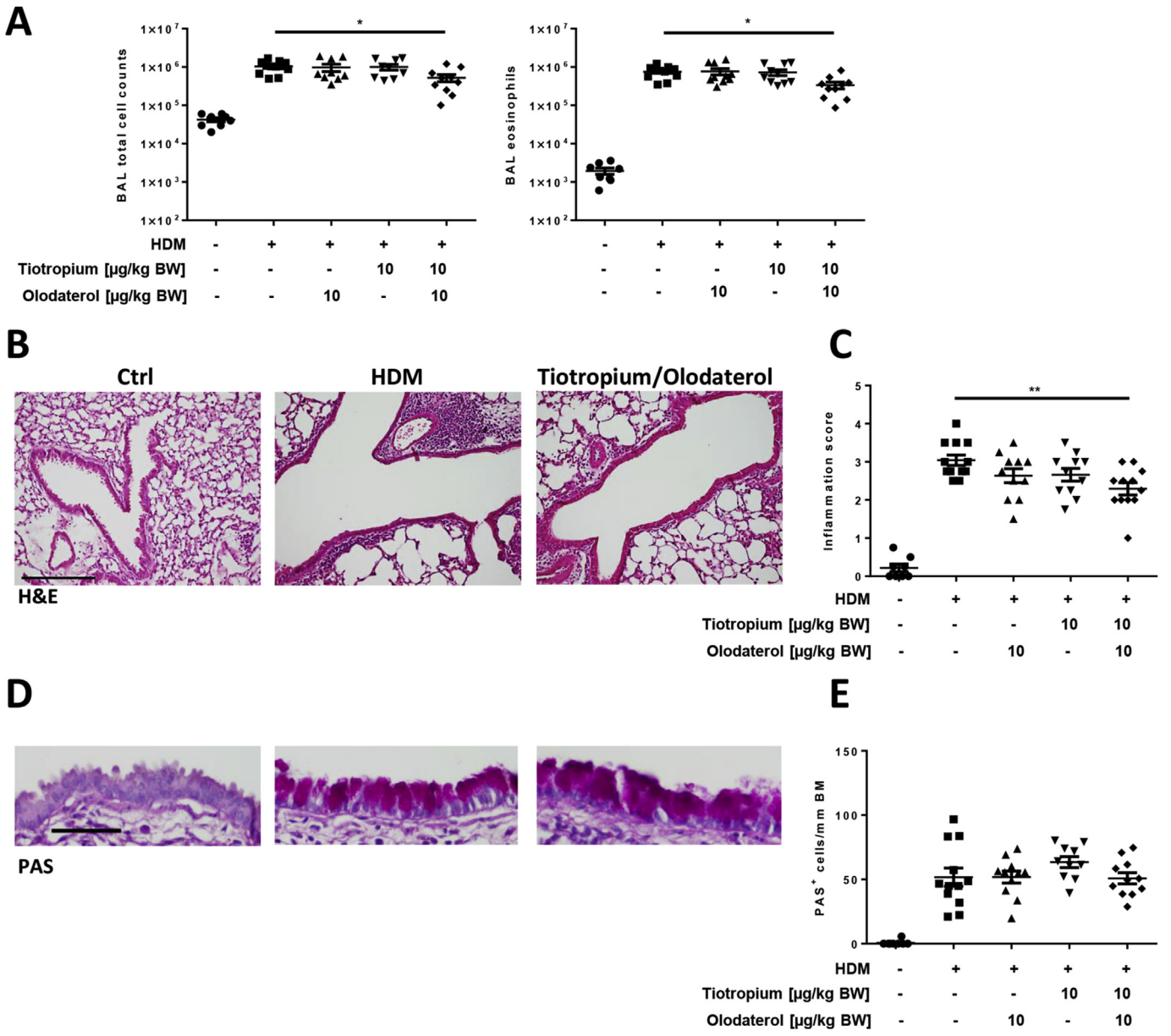

Fig. 4. A combination of tiotropium and olodaterol synergistically reduces allergic airway inflammation after allergen rechallenge. Mice were treated with a combination of tiotropium (10 µg/kg BW) and olodaterol (10 µg/kg BW) prior to and during the second allergen challenge period with HDM. **A** Total inflammatory cells in the BAL fluid were determined in a hemocytometer; absolute number of eosinophils were measured using FACS analysis; **B** representative micrographs of H&E-stained lung tissue sections from naïve, HDM allergic and HDM allergic mice treated with tiotropium/olodaterol, scale bar 200 µm; **C** quantification of lung tissue inflammation; **D** representative micrographs of PAS-stained lung tissue sections from naïve, HDM allergic and HDM allergic mice treated with tiotropium/olodaterol, scale bar 50 µm; **E** number of PAS-positive cells per mm of basal membrane. Graphs show mean ± SEM; n = 12 mice; *p < 0.05.

et al. also concluded that olodaterol-mediated cAMP signaling already provided a strong negative signal for fibroblast inflammatory responses including the production and release of pro-inflammatory cytokines and chemokines. This is in contrast to the generally accepted concept that LABAs are devoid of any clinically meaningful anti-inflammatory activity *in vivo* [43,44], which we also confirm in our rechallenge model with olodaterol treatment alone.

Nevertheless, the described mechanism could also explain the findings obtained in our study, where a lower dose of tiotropium in combination with olodaterol inhibited inflammation but not remodeling. In line with this, Buels et al. showed that OVA-induced airway hyperreactivity in guinea-pigs was prevented by a lower dose of tiotropium that was unable to inhibit vagally-induced bronchoconstriction, most likely related to a potential anti-inflammatory mechanism exerted by the lower dose of tiotropium as shown by inhibition of eosinophil accumulation in the lungs and around nerves [27]. These data suggest that anti-inflammatory actions of tiotropium are apparent at lower doses than are required for bronchodilation and might thus precede the effects on remodeling and bronchoconstriction. This is further supported by the above-mentioned study by Smit et al. who found that tiotropium and olodaterol treatment at relatively high concentrations inhibited OVA-induced bronchoconstriction but had no effect on inflammatory cell infiltration in the airways [16].

In summary, the present data show an effect of tiotropium treatment on the development of allergic airway inflammation in a murine model of allergen rechallenge using the human relevant

allergen HDM. Treatment with olodaterol did not inhibit the development of airway inflammation, however in combination with tiotropium it synergistically reduced recurrent allergic airway inflammation induced by allergen rechallenge. Tiotropium is increasingly utilized in human asthma treatment and has been associated with a reduction of acute exacerbations in asthma patients [45]. Whether treatment with tiotropium alone and in combination with olodaterol is also effective in reducing chronic inflammation needs to be assessed in further studies both in animal models of the disease and in patients with asthma.

Funding

This work was supported by an unrestricted research collaboration grant from Boehringer Ingelheim (Biberach, Germany).

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The authors thank the members of the Laboratory for Respiratory Cell Biology and Immunology of the Department of Pulmonology (LUMC) for their technical assistance.

References

[1] Global Initiative for Asthma, Global Strategy for Asthma Management and Prevention, 2016. Available from: www.ginasthma.org.
[2] H.E. Trejo Bittar, S.A. Yousem, S.E. Wenzel, Pathobiology of severe asthma, Annu. Rev. Pathol. 10 (2015) 511–545.
[3] T. Koumis, S. Samuel, Tiotropium bromide: a new long-acting bronchodilator for the treatment of chronic obstructive pulmonary disease, Clin. Ther. 27 (4) (2005) 377–392.
[4] D.P. Tashkin, B. Celli, S. Senn, D. Burkhart, S. Kesten, S. Menjoge, M. Decramer, U.S. Investigators, A 4-year trial of tiotropium in chronic obstructive pulmonary disease, N. Engl. J. Med. 359 (15) (2008) 1543–1554.
[5] J. Vestbo, S.S. Hurd, A.G. Agusti, P.W. Jones, C. Vogelmeier, A. Anzueto, P.J. Barnes, L.M. Fabbri, F.J. Martinez, M. Nishimura, R.A. Stockley, D.D. Sin, R. Rodriguez-Roisin, Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary, Am. J. Respir. Crit. Care Med. 187 (4) (2013) 347–365.
[6] P.J. Barnes, The pharmacological properties of tiotropium, Chest 117 (2 Suppl) (2000), 63S-6S.
[7] L.E. Kistemaker, R. Gosens, Acetylcholine beyond bronchoconstriction: roles in inflammation and remodeling, Trends Pharmacol. Sci. 36 (3) (2015) 164–171.
[8] J. Zaagsma, A.F. Roffel, H. Meurs, Muscarinic control of airway function, Life Sci. 60 (13–14) (1997) 1061–1068.
[9] N. Struckmann, S. Schwering, S. Wiegand, A. Gschnell, M. Yamada, W. Kummer, J. Wess, R.V. Haberberger, Role of muscarinic receptor subtypes in the constriction of peripheral airways: studies on receptor-deficient mice, Mol. Pharmacol. 64 (6) (2003) 1444–1451.
[10] S.I. Ramnarine, E.B. Haddad, A.M. Khawaja, J.C. Mak, D.F. Rogers, On muscarinic control of neurogenic mucus secretion in ferret trachea, J. Physiol. 494 (Pt 2) (1996) 577–586.
[11] D.F. Rogers, Motor control of airway goblet cells and glands, Respir. Physiol. 125 (1–2) (2001) 129–144.
[12] K.S. Buels, A.D. Fryer, Muscarinic receptor antagonists: effects on pulmonary function, Handb. Exp. Pharmacol. 208 (2012) 317–341.
[13] S. Ohta, N. Oda, T. Yokoe, A. Tanaka, Y. Yamamoto, Y. Watanabe, K. Minoguchi, T. Ohnishi, T. Hirose, H. Nagase, K. Ohta, M. Adachi, Effect of tiotropium bromide on airway inflammation and remodelling in a mouse model of asthma, Clin. Exp. Allergy 40 (8) (2010) 1266–1275.
[14] J.Y. Kang, C.K. Rhee, J.S. Kim, C.K. Park, S.J. Kim, S.H. Lee, H.K. Yoon, S.S. Kwon, Y.K. Kim, S.Y. Lee, Effect of tiotropium bromide on airway remodeling in a chronic asthma model, Ann. Allergy Asthma Immunol. 109 (1) (2012) 29–35.
[15] B. Bosnjak, C. Tilp, C. Tomsic, G. Dekan, M.P. Pieper, K.J. Erb, M.M. Epstein, Tiotropium bromide inhibits relapsing allergic asthma in BALB/c mice, Pulm. Pharmacol. Ther. 27 (1) (2014) 44–51.
[16] M. Smit, A.B. Zuidhof, S.I. Bos, H. Maarsingh, R. Gosens, J. Zaagsma, H. Meurs, Bronchoprotection by olodaterol is synergistically enhanced by tiotropium in a Guinea pig model of allergic asthma, J. Pharmacol. Exp. Ther. 348 (2) (2014) 303–310.
[17] H.A. Kerstjens, M. Engel, R. Dahl, P. Paggiaro, E. Beck, M. Vandewalker, R. Sigmund, W. Seibold, P. Moroni-Zentgraf, E.D. Bateman, Tiotropium in asthma poorly controlled with standard combination therapy, N. Engl. J. Med. 367 (13) (2012) 1198–1207.
[18] L. Costa, M. Roth, N. Miglino, L. Keglowich, J. Zhong, D. Lardinois, M. Tamm, P. Borger, Tiotropium sustains the anti-inflammatory action of olodaterol via the cyclic AMP pathway, Pulm. Pharmacol. Ther. 27 (1) (2014) 29–37.
[19] S.P. Peters, S.J. Kunselman, N. Icitovic, W.C. Moore, R. Pascual, B.T. Ameredes, H.A. Boushey, W.J. Calhoun, M. Castro, R.M. Cherniack, T. Craig, L. Denlinger, L.L. Engle, E.A. DiMango, J.V. Fahy, E. Israel, N. Jarjour, S.D. Kazani, M. Kraft, S.C. Lazarus, R.F. Lemanske Jr., N. Lugogo, R.J. Martin, D.A. Meyers, J. Ramsdell, C.A. Sorkness, E.R. Sutherland, S.J. Szefler, S.I. Wasserman, M.J. Walter, M.E. Wechsler, V.M. Chinchilli, E.R. Bleecker, L. National Heart, N. Blood Institute Asthma Clinical Research, Tiotropium bromide step-up therapy for adults with uncontrolled asthma, N. Engl. J. Med. 363 (18) (2010) 1715–1726.
[20] E.D. Bateman, O. Kornmann, P. Schmidt, A. Pivovarova, M. Engel, L.M. Fabbri, Tiotropium is noninferior to salmeterol in maintaining improved lung function in B16-Arg/Arg patients with asthma, J. Allergy Clin. Immunol. 128 (2) (2011) 315–322.
[21] H.A. Kerstjens, T.B. Casale, E.R. Bleecker, E.O. Meltzer, E. Pizzichini, O. Schmidt, M. Engel, L. Bour, C.B. Verkleij, P. Moroni-Zentgraf, E.D. Bateman, Tiotropium or salmeterol as add-on therapy to inhaled corticosteroids for patients with moderate symptomatic asthma: two replicate, double-blind, placebo-controlled, parallel-group, active-comparator, randomised trials, Lancet Respir. Med. 3 (5) (2015) 367–376.
[22] H.A. Kerstjens, B. Disse, W. Schroder-Babo, T.A. Bantje, M. Gahlemann, R. Sigmund, M. Engel, J.A. van Noord, Tiotropium improves lung function in patients with severe uncontrolled asthma: a randomized controlled trial, J. Allergy Clin. Immunol. 128 (2) (2011) 308–314.
[23] H.K. Reddel, E.D. Bateman, A. Becker, L.P. Boulet, A.A. Cruz, J.M. Drazen, T. Haahtela, S.S. Hurd, H. Inoue, J.C. de Jongste, R.F. Lemanske Jr., M.L. Levy, P.M. O'Byrne, P. Paggiaro, S.E. Pedersen, E. Pizzichini, M. Soto-Quiroz, S.J. Szefler, G.W. Wong, J.M. FitzGerald, A summary of the new GINA strategy: a roadmap to asthma control, Eur. Respir. J. 46 (3) (2015) 622–639.
[24] K. McKeage, Tiotropium respimat(R): a review of its use in asthma poorly controlled with inhaled corticosteroids and long-acting beta2-adrenergic agonists, Drugs 75 (7) (2015) 809–816.
[25] R. Gosens, I.S. Bos, J. Zaagsma, H. Meurs, Protective effects of tiotropium bromide in the progression of airway smooth muscle remodeling, Am. J. Respir. Crit. Care Med. 171 (10) (2005) 1096–1102.
[26] I.S. Bos, R. Gosens, A.B. Zuidhof, D. Schaafsma, A.J. Halayko, H. Meurs, J. Zaagsma, Inhibition of allergen-induced airway remodelling by tiotropium and budesonide: a comparison, Eur. Respir. J. 30 (4) (2007) 653–661.
[27] K.S. Buels, D.B. Jacoby, A.D. Fryer, Non-bronchodilating mechanisms of tiotropium prevent airway hyperreactivity in a Guinea-pig model of allergic asthma, Br. J. Pharmacol. 165 (5) (2012) 1501–1514.
[28] J.R. Johnson, R.E. Wiley, R. Fattouh, F.K. Swirski, B.U. Gajewska, A.J. Coyle, J.C. Gutierrez-Ramos, R. Ellis, M.D. Inman, M. Jordana, Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling, Am. J. Respir. Crit. Care Med. 169 (3) (2004) 378–385.
[29] M. Profita, A. Bonanno, L. Siena, M. Ferraro, A.M. Montalbano, F. Pompeo, L. Riccobono, M.P. Pieper, M. Gjomarkaj, Acetylcholine mediates the release of IL-8 in human bronchial epithelial cells by a NFkB/ERK-dependent mechanism, Eur. J. Pharmacol. 582 (1–3) (2008) 145–153.
[30] M. Profita, A. Bonanno, A.M. Montalbano, M. Ferraro, L. Siena, A. Bruno, S. Girbino, G.D. Albano, P. Casarosa, M.P. Pieper, M. Gjomarkaj, Cigarette smoke extract activates human bronchial epithelial cells affecting non-neuronal cholinergic system signalling in vitro, Life Sci. 89 (1–2) (2011) 36–43.
[31] L.E. Kistemaker, T.A. Oenema, H. Meurs, R. Gosens, Regulation of airway inflammation and remodeling by muscarinic receptors: perspectives on anticholinergic therapy in asthma and COPD, Life Sci. 91 (21–22) (2012) 1126–1133.
[32] C.L. Grainge, L.C. Lau, J.A. Ward, V. Dulay, G. Lahiff, S. Wilson, S. Holgate, D.E. Davies, P.H. Howarth, Effect of bronchoconstriction on airway remodeling in asthma, N. Engl. J. Med. 364 (21) (2011) 2006–2015.
[33] L.E. Kistemaker, S.T. Bos, W.M. Mudde, M.N. Hylkema, P.S. Hiemstra, J. Wess, H. Meurs, H.A. Kerstjens, R. Gosens, Muscarinic M(3) receptors contribute to allergen-induced airway remodeling in mice, Am. J. Respir. Cell Mol. Biol. 50 (4) (2014) 690–698.
[34] L.E. Kistemaker, P.S. Hiemstra, I.S. Bos, S. Bouwman, M. van den Berge, M.N. Hylkema, H. Meurs, H.A. Kerstjens, R. Gosens, Tiotropium attenuates IL-13-induced goblet cell metaplasia of human airway epithelial cells, Thorax 70 (7) (2015) 668–676.
[35] A. Gibb, L.P. Yang, Olodaterol: first global approval, Drugs 73 (16) (2013) 1841–1846.
[36] G. Pelaia, A. Vatrella, M.T. Busceti, L. Gallelli, C. Calabrese, R. Terracciano, N. Lombardo, R. Maselli, Pharmacologic rationale underlying the therapeutic effects of tiotropium/olodaterol in COPD, Ther. Clin. Risk Manag. 11 (2015) 1563–1572.
[37] M. Cazzola, P. Rogliani, J. Ora, M.G. Matera, Olodaterol + tiotropium bromide for the treatment of chronic obstructive pulmonary disease, Expert Rev. Clin. Pharmacol. 8 (5) (2015) 529–539.
[38] R. ZuWallack, L. Allen, G. Hernandez, N. Ting, R. Abrahams, Efficacy and safety of combining olodaterol Respimat((R)) and tiotropium HandiHaler((R)) in patients with COPD: results of two randomized, double-blind, active-controlled studies, Int. J. Chron. Obstruct Pulmon Dis. 9 (2014) 1133–1144.

[39] R. Buhl, F. Maltais, R. Abrahams, L. Bjermer, E. Derom, G. Ferguson, M. Flezar, J. Hebert, L. McGarvey, E. Pizzichini, J. Reid, A. Veale, L. Gronke, A. Hamilton, L. Korducki, K. Tetzlaff, S. Waitere-Wijker, H. Watz, E. Bateman, Tiotropium and olodaterol fixed-dose combination versus mono-components in COPD (GOLD 2-4), Eur. Respir. J. 45 (4) (2015) 969–979.
[40] K.M. Beeh, J. Westerman, A.M. Kirsten, J. Hebert, L. Gronke, A. Hamilton, K. Tetzlaff, E. Derom, The 24-h lung-function profile of once-daily tiotropium and olodaterol fixed-dose combination in chronic obstructive pulmonary disease, Pulm. Pharmacol. Ther. 32 (2015) 53–59.
[41] Tiotropium/olodaterol (Stiolto Respimat) for COPD, Med. Lett. Drugs Ther. 57 (1482) (2015) 161–162.
[42] J.F. Mosley 2nd, L.L. Smith, B.N. Dutton, Tiotropium bromide/olodaterol (Stiolto Respimat): once-daily combination therapy for the maintenance of COPD, P T 41 (2) (2016) 97–102.
[43] M. Cazzola, C.P. Page, P. Rogliani, M.G. Matera, beta2-agonist therapy in lung disease, Am. J. Respir. Crit. Care Med. 187 (7) (2013) 690–696.
[44] M. Cazzola, M.G. Matera, Safety of long-acting beta2-agonists in the treatment of asthma, Ther. Adv. Respir. Dis. 1 (1) (2007) 35–46.
[45] H. Matsuse, T. Yamagishi, N. Kodaka, A. Miura, Y. Kurose, C. Nakano, T. Oshio, Tiotropium bromide as add-on therapy to inhaled corticosteroids for treating asthma, Expert Opin. Pharmacother. 16 (9) (2015) 1403–1409.